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The DNA binding domains of the varicella-zoster virus gene 62 and herpes simplex virus type 1 ICP4 transactivator proteins heterodimerize and bind to DNA

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ABSTRACT

The product of varicella-zoster virus gene 62 (VZV 140k) is the functional counterpart of the major transcriptional regulatory protein of herpes simplex virus type 1 (HSV-1), ICP4. We have found that the purified bacterially expressed DNA binding domain of VZV 140k (residues 417–647) is a stable dimer in solution. As demonstrated by the appearance of a novel protein–DNA complex of intermediate mobility in gel retardation assays, following *in vitro* co-translation of a pair of differently sized VZV 140k DNA binding domain peptides, the 140k DNA binding domain peptide binds to DNA as a dimer. In addition, the DNA binding domain peptide of HSV-1 ICP4 readily heterodimerizes with the VZV 140k peptide on co-translation, indicating that HSV-1 ICP4 and VZV 140k possess very similar dimerization interfaces. It appears that only one fully wild type subunit of the dimer is sufficient to mediate sequence specific DNA recognition in certain circumstances. Co-immunoprecipitation analysis of mutant DNA binding domain peptides, co-translated with an epitope-tagged ICP4 DNA binding domain, shows that the sequence requirements for dimerization are lower than those necessary for DNA binding.

INTRODUCTION

Varicella-zoster virus is the causative agent of chickenpox on initial exposure and shingles on subsequent reactivation from latency. It is a member of the neurotropic alphaherpesvirinae subfamily and is genetically closely related to herpes simplex virus type 1 (1,2). Studies on the mechanisms of gene regulation during VZV infection are at an early stage, principally as a result of the poor growth properties of VZV in tissue culture. Much of our current understanding of alphaherpesvirus gene regulation has been derived from the more intensively studied HSV-1 prototype system. To date, 75 HSV-1 transcription units have been defined and are expressed in a complex temporally regulated cascade. The HSV-1 genes can be divided into three broad classes dependent on their kinetics of expression and the response to inhibitors of macromolecular synthesis. These are classified as

immediate-early (IE), early and late (for reviews, see 3,4). On HSV-1 infection, the first genes to be expressed in advance of any viral protein synthesis are the five IE genes, the products of at least three of which are involved in the transcriptional regulation of the later classes of HSV-1 genes (5). The product of the HSV-1 IE3 gene, ICP4, is essential for the activation of early and late gene transcription (6–9) and is also required for the autoregulation of its own promoter (10–12) and is therefore considered to be the major transcriptional regulatory protein of HSV-1.

Four varicella-zoster virus proteins have been detected in cells infected in the presence of cycloheximide (13) and as such they appear to be regulated in a fashion analogous to the HSV IE genes. All four have sequence homologues among the HSV-1 IE proteins (1). The product of VZV gene 62 has a predicted Mr of 140,000D (VZV 140k) and is the homologue of HSV-1 ICP4 on the basis of sequence homology and its position within the viral genome. Moreover, there is firm evidence that the VZV 140k protein is the functional counterpart of HSV-1 ICP4, as 140k can complement HSV-1 mutants lacking functional ICP4 (14–16). In transient transfection assays, VZV 140k strongly transactivates expression of VZV putative early and late gene promoters, and selected heterologous viral and cellular promoters (17–21). In transient tissue culture experiments, 140k either negatively autoregulates or transactivates its own gene 62 promoter, depending on the cell type used (22,23). Although there is no definite proof for an essential role of 140k in VZV biology, the functional similarity to the indispensable ICP4 protein of HSV-1 certainly argues for an important role in the VZV life cycle.

The primary sequence of VZV 140k has been divided into five regions on the basis of amino acid similarity to the homologous HSV-1 ICP4 protein (24). Protein regions 2 and 4 are particularly highly conserved and were predicted to have functional importance. Indeed, mutations within these regions are deleterious to the regulatory functions of HSV-1 ICP4. In particular, those within region 2 affected DNA binding, transactivation and promoter repression (25–27). Similarly, an insertion mutation in region 2 of VZV 140k inhibits the transactivation and repression phenotypes of 140k in transient transfection assays

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(22). The DNA binding domains of both the VZV 140k and the HSV-1 ICP4 protein have been found to consist mainly of the highly conserved region 2 sequences. Sequences spanning region 2 of each protein yield a stable, physically separable (28), DNA binding domain when expressed in isolation in bacteria (29–32). At least for ICP4, the isolated DNA binding domain has a DNA binding specificity very similar to the intact protein (33). The ICP4 protein recognizes a range of DNA binding sites, many of which conform broadly to the consensus ATCGT-CnnnnYCGRC (34). The 140k DNA binding domain is less sequence specific than ICP4 and this low specificity of binding allows the recognition of multiple sequences within target promoters (32). Because an antigenic epitope spanning the boundary between regions 1 and 2 is conserved in both VZV 140k and HSV-1 ICP4, it is likely that at least some similar structural features are shared between the DNA binding domains of the two proteins (35).

As is the case for many other DNA binding proteins, the native form of the HSV-1 ICP4 protein is homodimeric (36,37) and evidence suggests it binds to DNA as a multimer (38), and the stoichiometry appeared to be two monomers per binding site (39). In addition, the isolated ICP4 DNA binding domain peptide exists as a stable dimer in solution (33), suggesting that sequences overlapping the DNA binding domain are involved in the dimerization of the intact ICP4 protein. Although the VZV 140k protein appears to share many common features with its HSV-1 counterpart, numerous variations have been identified in the details of their regulatory functions (21,22) and the DNA binding characteristics of their isolated DNA binding domains (32). We sought to establish whether the similarities between these two proteins extended to their oligomeric state and the sequences involved in the formation of possible higher order complexes.

In this study we report that the DNA binding domain of the VZV 140k protein is a stable dimer in solution, and we demonstrate that the 140k DNA binding domain interacts with its DNA recognition sites as a dimer. In addition, we have extended the similarities previously recognised between the VZV 140k and HSV-1 ICP4 proteins to include structural similarities; the two proteins must have very similar dimerization interfaces as heterodimer formation readily occurs between their corresponding DNA binding domain peptides. Interestingly, these heterodimers can interact with DNA sequences to which the HSV-1 ICP4 DNA binding domain homodimers do not bind, suggesting that only one fully wild type subunit of the dimer is sufficient for specific DNA binding. Finally, we present data to show that the dimerization activity of these homologous alphaherpesvirus DNA binding domains has a lower protein sequence requirement and is less mutation sensitive than the DNA binding function.

MATERIALS AND METHODS

Plasmids and bacteria

Plasmid p585T7a is a T7 expression vector derived from pET-8c (40), which includes a multiple cloning region preceded by an ATG start codon (32). DNA fragments encompassing the highly conserved region 2 (Fig. 1) of VZV gene 62 were inserted into the p585T7a expression vector, as detailed below:

Expression plasmids p585T7aVT2 and p585T7aVT2X contain the VZV gene 62 sequences encoding residues 417–647 as described (32). The products of plasmids p585T7aVT2 and p585T7aVT2X are referred to as VT2 (256 residues, Mr

–28.4kD) and VT2X (240 residues, Mr –26.7kD) respectively (Fig. 1). Plasmid p585T7aVT4 was constructed by inserting the *Bsp*HI–*Bam*HI fragment of p585T7aVT2 (encompassing gene 62 codons 462–647) into the complementary *Nco*I and *Bam*HI sites of plasmid p585T7a. Plasmid p585T7aVT4X was derived from p585T7aVT4 by insertion of an oligonucleotide, containing stop codons in all three reading frames, into the *Bam*HI site at the 3' end of the VZV gene 62 coding sequences. The product of p585T7aVT4X, termed VT4X (191 residues, Mr –21.2kD), comprises an N-terminal vector-encoded methionine residue preceding VZV 140k residues 462–647, followed by five C-terminal residues (GDQLI) encoded by vector sequences 3' to the insert. Plasmids expressing peptides VT5 and VT11 were constructed using appropriate restriction enzyme sites in the gene 62 sequences (32). Similarly, plasmids expressing the VT8 and VT12 peptides included the *Eco*RI–*Nco*I fragment of plasmid pC34 and the *Rsa*I–*Kpn*I fragment of plasmid p585T7aVT2, respectively, cloned into p585T7 expression vectors with an appropriate linker to maintain the reading frame. The gene 62 coding regions included in each 140k DNA binding domain deletion peptide are given in Figure 7A.

Plasmids pT719X and pT7110X include sequences encompassing the HSV-1 ICP4 DNA binding domain as described (30). The products of plasmids pT719X and pT7110X are referred to as I9X and I10X respectively (Fig. 1). Plasmids pI12–I19 are derivatives of plasmid pI75 containing 4 codon insertions into the ICP4 coding sequence as described (25). The *Nru*I–*Bam*HI fragments carrying the i12 to i19 insertion mutations were isolated from plasmids pI12–pI19 and cloned into the *Nru*I–*Bam*HI sites of plasmid pJ110X (a p585T7a derivative of pT7110X). The resulting plasmids, pT7110i12X–i19X encoded insertion mutation versions of the I10X ICP4 DNA binding domain (Fig. 6A).

Plasmid pNXI10X encodes a fusion protein comprising residues 1–105 of HSV-1 ICP0, fused to the N-terminal end of the I10X peptide (Fig. 6A). It was constructed by ligating the *Eco*RI–*Pst*I fragment of plasmid pJ110X (including ICP4 codons 275–523) to the *Pst*I–*Xho*I fragment (including ICP0 codons 1–105) of plasmid pT7110 (41), with the aid of an *Xho*I–*Eco*RI adaptor which maintains the translational reading frame. The product of plasmid pNXI10X is termed NXI10X; translation begins at the first ATG of the HSV-1 IE1 gene, and a single vector-encoded residue is at the C-terminal end of the NXI10X fusion peptide (predicted size of 40kD).

All plasmids were constructed and maintained in *E. coli* strain DH5 α . Their authenticity was confirmed by extensive restriction enzyme analysis and direct DNA sequencing. Plasmids were transformed into *E. coli* strain BL21 (DE3) pLysS (40) for the bacterial expression experiments.

Bacterial expression and purification of the VZV 140k DNA binding domain

E. coli transformed with p585T7aVT2 were grown up, induced, harvested and crude bacterial extracts prepared exactly as described (32). The VZV 140k DNA binding domain was purified from the initial 35% ammonium sulphate precipitated extract by two steps of Pharmacia FPLC ion exchange chromatography, followed by a gel filtration step. The following buffer was used for all column elutions at the salt concentrations specified: 50mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.5), 1mM phenylmethylsulfonyl fluoride (PMSF), 0.1mM dithiothreitol (DTT), 0.01%

2-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The first step used a 20ml Mono Q column equilibrated in 0.1M NaCl buffer. 1ml of crude extract from a one litre bacterial culture was diluted to 5ml in 0.1M NaCl buffer and loaded onto the column and eluted with a linear 0.1 to 1M NaCl salt gradient. The VT2 peptide eluted in the flow-through. Fractions containing VT2 were detected by Coomassie-staining of SDS-polyacrylamide gels at this and subsequent stages of the purification. Pooled Mono Q flow-through fractions were loaded onto a 20ml Mono S column equilibrated in 0.25M NaCl buffer. A 0.25M to 0.45M NaCl salt gradient eluted the VT2 peptide at approximately 0.38M NaCl. Peak fractions were concentrated by centrifugation in Centricon 10 filtration units (Amicon). 250 μ l of the concentrated VT2 sample was loaded onto a Superose 12 column equilibrated and eluted in 0.38M NaCl buffer. VT2 eluted in a single sharp peak and peak fractions were pooled and concentrated to yield a preparation of VT2 of apparent homogeneity on SDS PAGE gels. The preservation of DNA binding activity was verified by gel retardation analysis of aliquots of the VT2 peptide at each stage of the purification procedure (data not shown).

Protein cross-linking

Incubation with glutaraldehyde is a standard method used to cross link covalently α NH₂ groups and the ϵ -amino groups of lysine residues of proteins in close proximity i.e. the monomers of an oligomeric complex (42). 4 μ g of either purified VT2 peptide or bovine carbonic anhydrase (Sigma) was incubated in a buffer containing 50mM HEPES (pH 7.5), 0.1M NaCl, 1mM PMSF either with or without 0.01% glutaraldehyde for 30min at 31°C. The products of the cross-linking were analyzed by electrophoresis through SDS-polyacrylamide gels using the Biorad miniprotein II kit and visualised by staining with Coomassie blue.

Gel filtration chromatography

The dimerization of the 140k DNA binding domain was determined by gel filtration analysis. A Pharmacia Superdex 75 column was equilibrated with elution buffer [50mM HEPES (pH 7.5), 0.3M NaCl, 1mM PMSF, 0.1mM DTT, 0.01% CHAPS] before loading either purified VT2 or gel filtration protein standards (Sigma) diluted to 200 μ l in the elution buffer. The eluted proteins were detected by the uv monitor and the identity of the protein peaks verified by SDS PAGE. The retention volume of each protein peak was provided by the software in the FPLC equipment.

In vitro coupled transcription/translation

The isolated HSV-1 ICP4 and VZV 140k DNA binding domains were transcribed and translated *in vitro* (TNT, Promega) in the presence of ³⁵S-methionine, following the manufacturer's instructions. T7 expression plasmids (described above) were transcribed and translated by TNT rabbit reticulocyte lysates either individually or in pairs. For co-translation experiments, the amount of each plasmid required to yield equivalent amounts of peptide was determined empirically. Typically, 0.012 μ g of plasmid p585T7aVT2X was used, as this yielded high levels of VT2X expression whilst minimising VT2X binding to the input plasmid DNA. Co-translation experiments contained half the amount of each plasmid used in the single translation experiments. Control translation reactions contained plasmid p585T7a. Within each set of experiments, the total amount of input plasmid DNA

was equalised with plasmid p585T7a. Translation reactions were incubated at 30°C for 90min. The translation products (1 μ l) were analysed by SDS PAGE and processed for autoradiography. *In vitro* translation reactions for co-immunoprecipitation analysis included 1 μ g of each plasmid DNA.

DNA fragment probes

End-labelled probes for gel retardation analysis were prepared as follows: The HSV-1 IE3 probe [including the strong consensus ICP4 binding site spanning the mRNA start site (43), which is also recognised by the 140k DNA binding domain peptides (32)] included IE3 sequences -18 to +27 and was isolated as an *Eco*RI-*Hind*III fragment from plasmid pUCIE3. Plasmid pUCIE3 has the *Ava*I(end filled)-*Bam*HI fragment of the IE3 gene inserted between the *Hinc*II and *Bam*HI sites of the PUC19 multiple cloning region. The pUCIE3 *Hind*III-*Eco*RI fragment was end-labelled with Klenow DNA polymerase and the probe eluted from an 8% acrylamide gel and purified by ethanol precipitation. The BT fragment probe (encompassing sequences -130 to +57 of the VZV gene 62 promoter which include numerous binding sites recognised by the 140k DNA binding domain) was prepared as described (32).

Gel retardation assay

Appropriate amounts of extracts were incubated on ice with 1ng of a DNA probe in buffer giving final concentrations of 10mM Tris·HCl (pH 8.0), 0.1mM EDTA, 100mM NaCl, 0.2% CHAPS, 5mM DTT. After 20min, 0.2 volumes of loading buffer [10mM Tris·HCl (pH 8.0), 1mM EDTA, 10mM 2-mercaptoethanol (BME), 0.1% CHAPS, 50% glycerol and 0.25mg/ml bromophenol blue] was added. Samples were applied to a non-denaturing 4% polyacrylamide gel with 0.5 \times TBE running buffer and run at 150V at 4°C for 3hr. Due to the relatively small difference between the sizes of peptides VT2X and VT4X, samples from the VT2X/VT4X co-translation experiments were applied to 35cm gels and run for 40hr. Protein-DNA complexes were detected by autoradiography of dried gels.

Co-immunoprecipitation of in vitro translated proteins

DNA binding domain peptides were co-immunoprecipitated after co-translation *in vitro* with fusion peptide NX110X, which includes an HSV-1 ICP0 epitope recognised by MAb 11,060 (44,45). TNT translation reactions (50 μ l) were pre-cleared by incubation for 30min at 4°C with 10vol IP buffer (0.05M Tris·HCl (pH7.5), 0.3M NaCl₂, 1mM EDTA, 0.2% NP40) containing 5 μ l normal mouse serum, 12.5 μ l sheep anti-mouse Ig (S α M) and 60 μ l 50% slurry of protein A sepharose beads. After a 2min spin, 20 μ l of the supernatant was incubated for 2hr at 4°C with 2 μ l MAb, 5 μ l S α M, 60 μ l 50% slurry protein A sepharose, in a volume made up to 400 μ l with IP buffer. Immune complexes were pelleted by centrifugation for 2min. The pellet was washed 3 times with IP buffer, and then washed with a buffer containing 0.6M LiCl, 0.1M Tris·HCl (pH 7.5), 1% BME. The pellet was finally washed in PBSA and resuspended in 20 μ l of a denaturing boiling mix and heated at 100°C for 5min. The beads were pelleted and the supernatant analysed by 12.5% SDS PAGE. After electrophoresis, the proteins were electroblotted to nitrocellulose membranes. Co-immunoprecipitated ³⁵S labelled proteins were detected by autoradiography, or phosphorimaging of the dried membranes using ImageQuant software (Molecular Dynamics). Protease inhibitors (Boehringer Mannheim) were

included throughout the procedure (bestatin, leupeptin, aprotinin, PMSF, at concentrations recommended by the manufacturer).

RESULTS

Bacterial expression and purification of the VZV 140k DNA binding domain peptide

The sequences of the VZV 140k polypeptide that are required for the DNA binding have been mapped to between residues 417 and 647 (32), which encompasses the whole of the highly

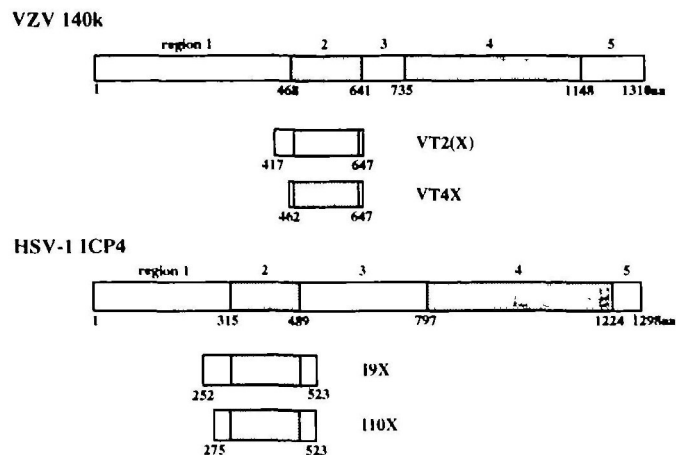


Figure 1. VZV 140k and HSV-1 ICP4 and their DNA binding domain peptides. Regions 2 and 4 are highly conserved (46% and 56% identity respectively) between the two proteins and are shown shaded, amino acid coordinates of the boundaries of the conserved regions are given (inclusively) (24). Regions 1, 3 and 5 are less highly conserved (15–25% identity). The VT2(X) and VT4X peptides encompass the 140k DNA binding domain and the equivalent 19X and 110X peptides (32) encompass the ICP4 DNA binding domain, the protein coding regions included in each peptide are indicated (inclusively).

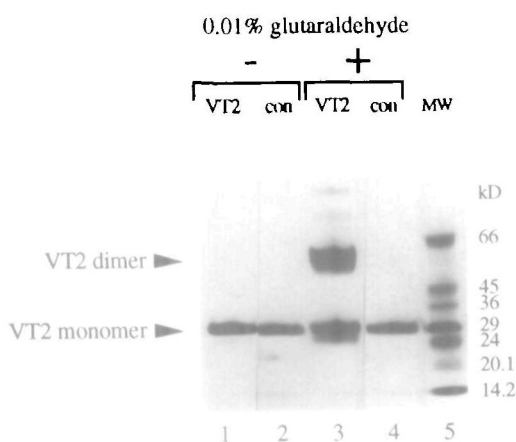


Figure 2. Glutaraldehyde cross-linking of the VZV 140k DNA binding domain. 12.5% SDS PAGE analysis of samples of purified VT2 peptide incubated with or without glutaraldehyde (see Materials and Methods). Tracks 2 and 4 contain an equivalent amount of the monomeric carbonic anhydrase protein (Mr = 29kD). Track 5 contains molecular weight markers: BSA, 66kD; albumin, 45kD; glyceraldehyde-3-phosphate dehydrogenase, 36kD; carbonic anhydrase 29kD; trypsinogen, 24kD; trypsin inhibitor, 20.1kD; lactalbumin, 14.2kD. The positions of VT2 monomer and dimer bands are indicated (calculated monomeric size, 28.4kD).

conserved region 2 and the C-terminal end of the relatively non-conserved region 1 of the protein. The cloning and expression of residues 417–647 of the VZV 140k coding region in *E. coli* using the T7 system has been described (32) and the resultant 28.4kD peptide is referred to as VT2 (Fig. 1). Another VZV 140k peptide essentially comprising region 2 (peptide VT4X—residues 462–647, Fig. 1), possesses a similar DNA binding affinity to the larger VT2 peptide as determined by gel retardation analysis, although VT4X fails to give a DNase I footprint (32). We chose to purify the VT2 peptide as we considered it more likely to reflect the characteristics of intact VZV 140k. VT2 was purified by ion-exchange and gel filtration chromatography to near homogeneity as described in the materials and methods section, obtaining yields of at least 10mg per litre of bacterial culture. Figure 2, track 1 shows an example of the Coomassie-stained final preparation of purified VT2.

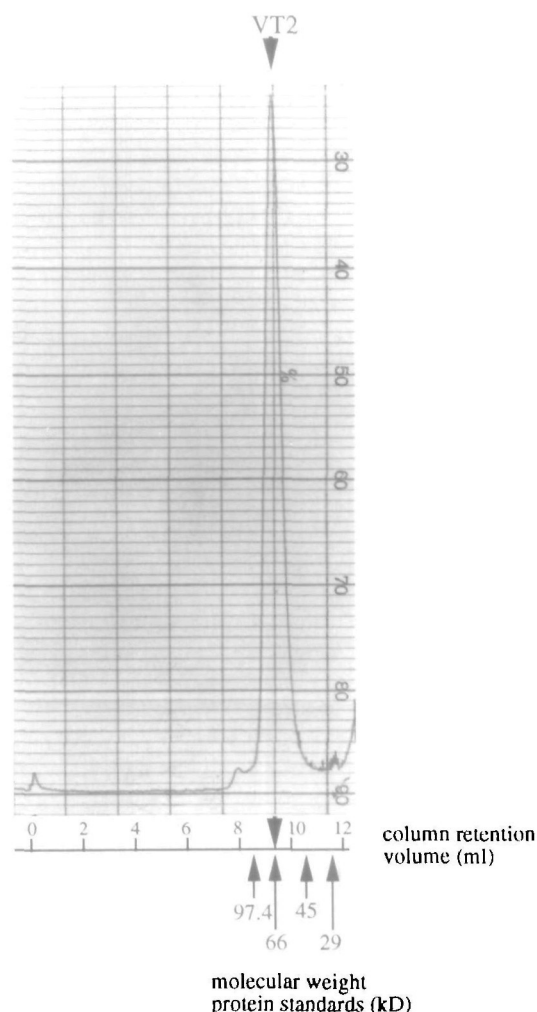


Figure 3. Gel filtration analysis of the VZV 140k DNA binding domain. The uv trace shows a Superdex 75 column elution of a purified sample of the VT2 peptide (see Materials and Methods). The scale indicates retention volumes corresponding to the uv trace. VT2 eluted with a retention volume of 9.3ml, retention volumes of gel filtration protein standards are also indicated: phosphorylase b, 97.4kD; BSA, 66kD; albumin, 45kD; carbonic anhydrase, 29kD. The native size of approximately 65kD for the VT2 peptide (predicted monomer size, 28.4kD) was derived from a calibration curve plotted for a wide range of protein standards (not shown).

The purified VZV 140k DNA binding domain is a dimer in solution

The native form of the intact HSV-1 ICP4 protein is dimeric (37), as is the isolated ICP4 DNA binding domain (33). By analogy, it seemed probable that the VT2 peptide would also be dimeric. To investigate this possibility, we incubated the purified VT2 peptide with the cross-linking agent glutaraldehyde and analysed the products by SDS PAGE. Figure 2, track 3 shows clear formation of VT2 dimers, not produced in the absence of glutaraldehyde (Fig. 2, track 1). The faint VT2 products of higher molecular weight than the dimers, which arise following incubation with glutaraldehyde, presumably reflect either non-specific cross-linking or a low level of higher order oligomers in the VT2 sample.

Having found that the 140k DNA binding domain has the ability to dimerize, we were interested to determine whether VT2 is a stable dimer in solution, or whether an equilibrium exists between VT2 monomers and dimers. An analytical gel filtration FPLC column (Superdex 75, Pharmacia) was equilibrated in a suitable buffer (see Materials and Methods) and the retention volumes of a range of protein standards of known molecular weights were determined. The purified VT2 peptide yielded a sharp peak having a retention volume consistent with it being a dimer in solution (Fig. 3). The failure to observe any peaks eluting at the VT2 monomer position suggested that VT2 dimers are not in equilibrium with monomers. Thus, the dimers appeared to be highly stable units. The dimeric nature of VT2 was verified using glycerol gradient centrifugation analysis to compare the sedimentation rates of purified VT2 with those of protein standards of known molecular weights loaded on the same

gradients (data not shown). From these data, it was apparent that the 140k DNA binding domain contains a dimerization surface and it is likely that the intact 140k protein is also dimeric. Whether the DNA binding domain region alone is sufficient for dimerization of the intact protein, or whether other protein sequences are additionally involved, remains to be determined.

The 140k DNA binding domain interacts with its DNA recognition sites as a dimer

We used the method introduced by Hope and Struhl (46) to test whether the 140k DNA binding domain peptide bound to its DNA recognition site in an oligomerized form. The radiolabelled peptides used for these analyses were produced by *in vitro* coupled transcription/translation reactions in rabbit reticulocyte lysates (see Materials and Methods). *In vitro* expression utilized the same T7 expression plasmids used previously for the bacterial expression (32) and the translation products were resolved by gel retardation analysis using the BT probe fragment derived from the VZV gene 62 promoter. The VT4X peptide, a DNA binding competent truncated version of VT2 (Fig. 1), was tested for its ability to form heterodimers in gel retardation assays with the larger VT2X peptide (the VT2X peptide contains the same 140k residues as VT2, but is more stable than the VT2 version) (32). When the VT2X peptide was incubated with the DNA fragment, it produced a slower-migrating species than did the VT4X truncation peptide in gel retardation analysis (Fig. 4A). When the two separately translated proteins were incubated together only two bands corresponding to the separate VT2X and VT4X protein-DNA complexes were observed. However, when the two proteins were co-translated, a third intermediate band was

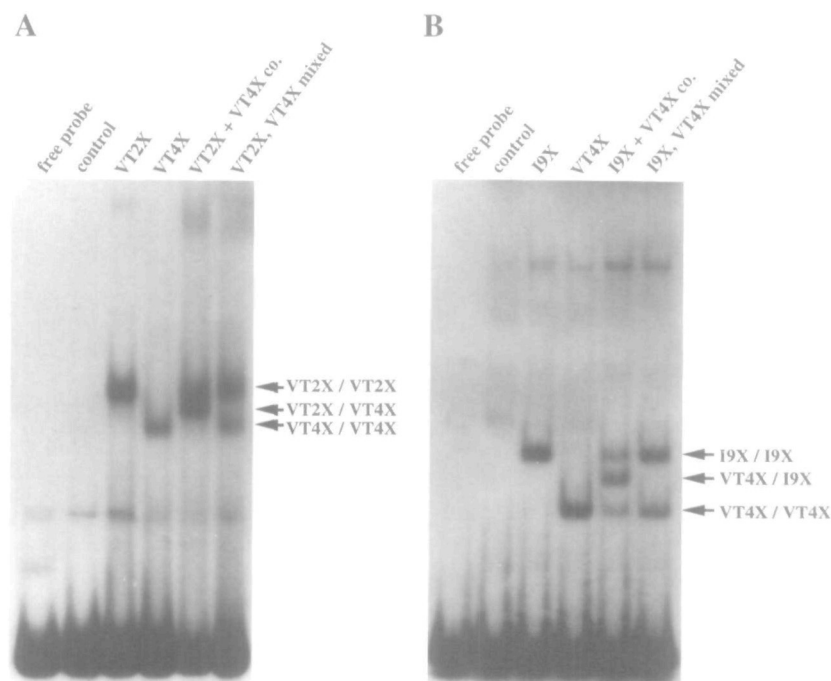


Figure 4. Gel retardation analysis of heterodimer formation. DNA probes were incubated with 1 μ l (unless otherwise stated) of the *in vitro* translated extracts indicated above each track (see Materials and Methods). Control translation reactions included plasmid p58ST7a. Incubations for the tracks labelled 'co.' contained 2 μ l of co-translation extracts. Incubations for tracks labelled 'mixed' contain 1 ml of each separately translated extract. **A.** Heterodimer formation between the 140k peptides VT2X and VT4X on the BT probe. **B.** Heterodimer formation between the VZV 140k peptide VT4X and the HSV-1 ICP4 peptide I9X on the IE3 probe. The protein regions included in each peptide are illustrated in Fig. 1. Dimer complexes are identified on the right of each panel. Faint non-specific bands due to TNT lysate proteins remain unlabelled.

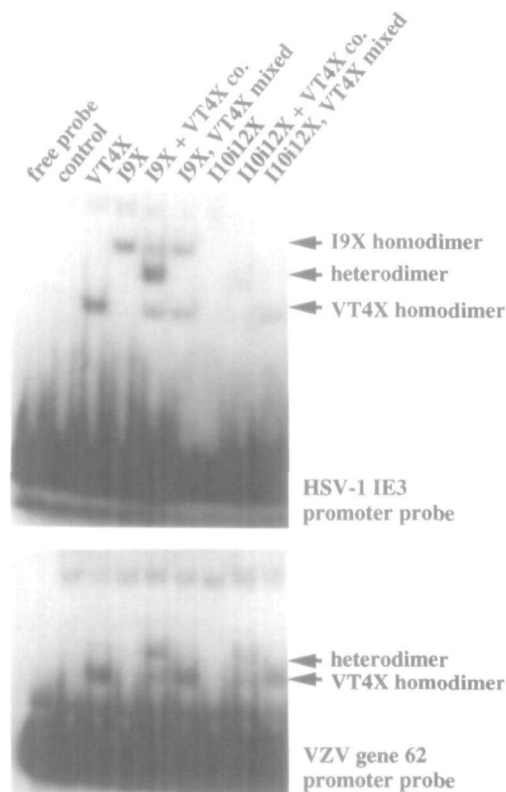


Figure 5. Gel retardation analysis of VZV 140k/HSV-1 ICP4 peptide heterodimers. Aliquots of extracts produced from a single set of *in vitro* translation reactions (as identified above the tracks) were incubated with either the IE3 probe (top) or the BT probe (bottom). The volumes of extracts used in the incubations were as described in the legend of Figure 4. The details of the VT4X, I9X and I10i12X peptides are given in Figs. 1 and 6A. Dimer complexes are identified on the right.

observed (Fig. 4A, 'VT2X + VT4X co.' track). We interpret this intermediate band to correspond to a heterodimer formed between the VT2X peptide and the smaller VT4X truncation peptide. The finding that the intermediate heterodimeric VT2X/VT4X band was not formed by mixing of the two peptides confirms that the dimers are highly stable, as there is no detectable exchange of monomer subunits within the 20 minute incubation period of the binding reaction.

The VZV 140k DNA binding domain heterodimerizes with the corresponding domain of HSV-1 ICP4

Given that the DNA binding domains of the VZV 140k and the HSV-1 ICP4 proteins are both dimeric and that their primary sequences are similar (Fig. 1), we thought it possible that they might dimerize in a similar manner. Therefore we investigated whether heterodimers could be produced between the HSV-1 ICP4 and VZV 140k DNA binding domains, using *in vitro* co-translation of peptide VT4X with the larger HSV-1 ICP4 peptide, I9X (Fig. 1) (30). The resulting DNA binding competent protein complexes were resolved by gel retardation analysis, using the HSV-1 IE3 probe, which is strongly recognised by both the VZV 140k and the HSV-1 ICP4 DNA binding domains (32). Figure 4B illustrates that a novel heterodimer band of intermediate

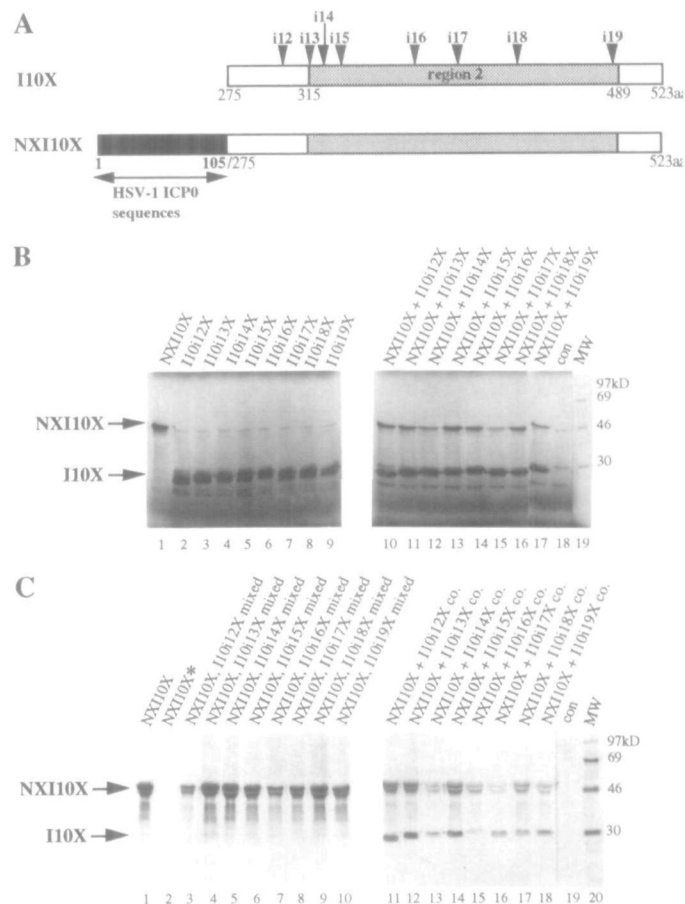


Figure 6. Co-immunoprecipitation analysis of the insertion mutation versions of the HSV-1 ICP4 DNA binding domain. **A.** Representations of the I10X peptide and its epitope-tagged derivative, NXI10X. The highly conserved ICP4 region 2 is indicated by light shading, the HSV-1 ICP0 sequences are indicated by dark shading. The arrows indicate the positions of the 4 amino acid insertions (25) introduced into the following ICP4 codons: insertion i12 at codon 310; i13 at 324; i14 at 329; i15 at 337; i16 at 373; i17 at 398; i18 at 438; i19 at 494. **B.** Pre-cleared *in vitro* translated extracts of the I10X insertion peptides and the NXI10X peptide. The I10i12X-I10i19X peptides were *in vitro* translated individually, or co-translated with NXI10X, followed by 'pre-clearing' to reduce background (see Materials and Methods). 20 μ l aliquots of pre-cleared extracts (as indicated above each track) were analysed by 12.5% SDS PAGE. 'con' indicates an unprogrammed translation reaction. 'MW' indicates 14 C radiolabelled molecular weight markers: phosphorylase b, 97kD, bovine serum albumin, 69kD; ovalbumin, 46kD; carbonic anhydrase, 30kD. The positions of peptides NXI10X (40kD) and I10X (28.2kD) are indicated on the left. The highly acidic nature of the ICP0 sequences reduced the mobility of the NXI10X peptide. **C.** Co-immunoprecipitation analysis of the I10X insertion peptides. The pre-cleared extracts shown in panel B were immunoprecipitated with MAb 11,060, * with the exception of track 2 which included MAb 10,503 as a negative control (44). Peptides included in the immunoprecipitation reactions were either co-translated or mixed after translation. Molecular weight markers are as above.

mobility was clearly produced upon co-translation of VT4X and I9X, and the relative amounts of the three complexes are in the expected 1:2:1 proportions. The VT4X/I9X heterodimer was not formed when the separately translated peptides were mixed after translation. This result suggests that the VZV 140k and HSV-1 ICP4 proteins have very similar structural requirements, and perhaps similar sequence requirements, for dimerization.

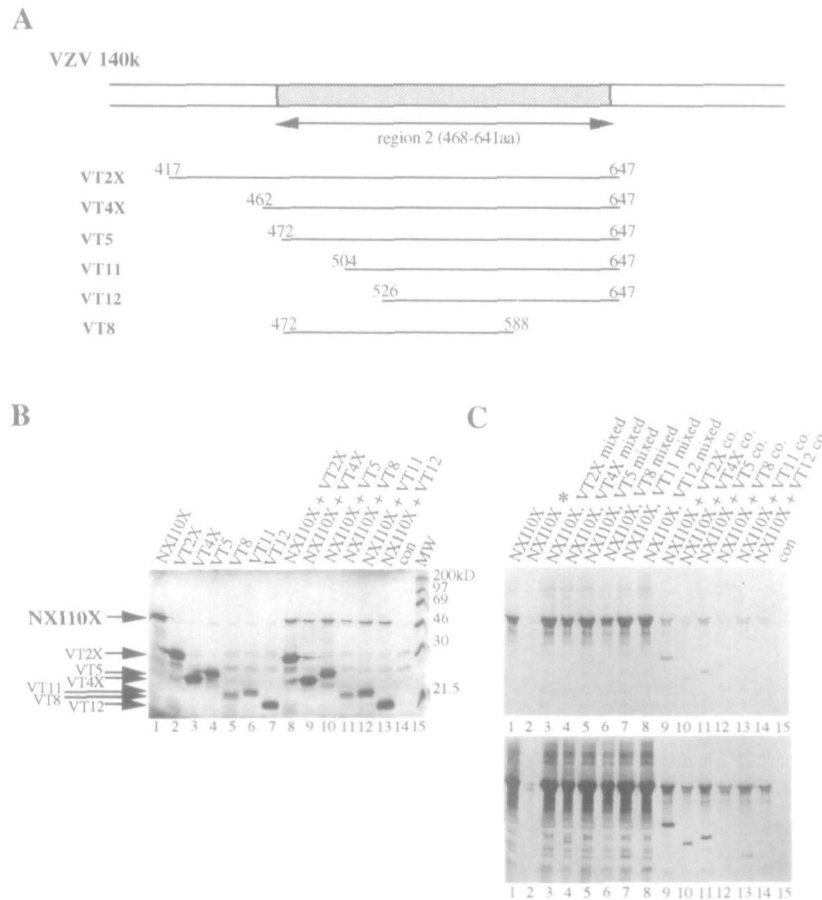


Figure 7. Co-immunoprecipitation analysis of the VZV 140k DNA binding domain deletion peptides. **A.** Schematic of the region spanning the DNA binding domain of the VZV 140k protein, the highly conserved region 2 is indicated by shading. Beneath, horizontal lines represent the 140k DNA binding domain deletion peptides of the VT series (32), the 140k amino acid residues included in each are given. The VT peptides were *in vitro* translated individually, or co-translated with the epitope-tagged NX110X peptide. The extracts were pre-cleared (panel B) and immunoprecipitated (panel C) as described in Figure 6. The relative positions of *in vitro* translated peptides are indicated on the far left. The greater amount of NX110X peptide in tracks 3–8 compared to tracks 9–14, panel C, is a direct consequence of the high amount of NX110X in track 1, panel B. Instability of the NX110X fusion peptide is apparent following long exposure of the filter, as shown in lower part of panel C. 'MW' indicates ^{14}C radiolabelled molecular weight markers: myosin, 200kD; phosphorylase b, 97kD; bovine serum albumin, 69kD; ovalbumin, 46kD; carbonic anhydrase, 30kD; trypsin inhibitor, 21.5kD; lysozyme, 14.3kD.

Selected heterodimers with only one fully wild type subunit can bind to DNA

The HSV-1 ICP4 protein DNA binding domain has a very low affinity for the 140k binding sites in the VZV gene 62 promoter (compared to its affinity for the IE3 probe) whereas the 140k peptide recognizes sites in the VZV gene 62 promoter and also the HSV-1 IE3 consensus binding site (32). In order to see whether both subunits of the dimer contribute to the sequence specificity of DNA binding, we tested whether *in vitro* produced heterodimers of the ICP4/140k DNA binding domains were able to recognise the VZV gene 62 promoter probe in gel retardation analysis. The top panel of Figure 5 confirms the result of Figure 4B and shows that both homodimers and the heterodimer bind to the IE3 probe. Aliquots of the same *in vitro* produced extracts were tested for DNA binding activities on the VZV gene 62 promoter fragment probe (Fig. 5, bottom panel). The very low affinity of the ICP4 DNA binding domain for this particular probe is seen by the lack of any specific bands in the I9X track. However the heterodimer clearly recognizes the gene 62 promoter binding sites, as a band in a position consistent with the production

of a heterodimer–DNA complex (compare with top panel of Fig. 5) is formed upon co-translation of I9X and VT4X. This result suggests that the specific interaction of the single VT4X monomer with its DNA recognition site is sufficient to mediate the DNA binding activity of the I9X/VT4X heterodimer. However, it is also possible that the interaction with VT4X induces a conformational change in the I9X subunit which allows interaction with normally unrecognised binding sites (see discussion).

We were interested to distinguish sequences within the DNA binding domain which specify dimerization and DNA recognition. The functional domains of the ICP4 protein were originally mapped by an insertion mutagenesis of the entire ICP4 coding region (25–27). Many of the insertions within the DNA binding domain prevented DNA binding of the intact ICP4 protein. It is possible that some of these mutations affect DNA binding indirectly, by interfering with dimerization. The VT4X/I9X heterodimer experiment suggests that, in certain circumstances, the specific DNA binding property of the heterodimer can be provided by the VT4X subunit alone. Therefore it is possible that VT4X heterodimers with altered forms of the I9X subunit might also bind to DNA. Such DNA binding competent mutant

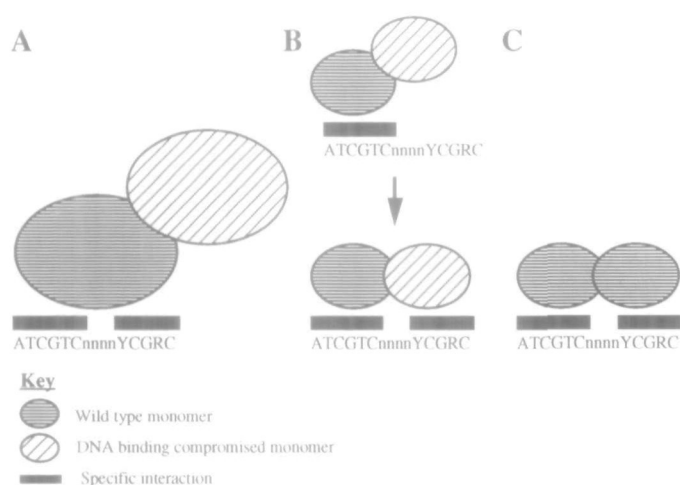


Figure 8. Models for DNA recognition by the ICP4/140k dimers. Each oval represents a DNA binding domain monomer. Each panel illustrates a possible mode of binding site recognition as discussed in the text, and the binding sequence represents the consensus ICP4 binding site where n = any nucleotide, Y = pyrimidine, R = purine (34). The dimer represented comprises a wild type monomer and a DNA binding compromised monomer (i.e. a monomer that has a low binding affinity for the binding site, or containing a mutation that is deleterious for DNA binding). **A.** Only one subunit of the dimer recognizes the ICP4 consensus binding site. **B.** Binding of one monomer facilitates binding of the second monomer. **C.** Heterodimerization induces a conformational change in the DNA binding compromised monomer, such that it adopts a wild type conformation that enables recognition of the binding site

heterodimers would identify positions which are involved in DNA binding rather than dimerization. Therefore, insertion mutation versions of the isolated ICP4 DNA binding domain (I10X) were *in vitro* co-translated with the VZV 140k DNA binding domain peptide (VT4X) and heterodimer formation assayed by gel retardation analysis.

The i12 insertion into codon 310 in the C-terminal end of region 1 of ICP4 (Fig. 6A) greatly reduces the DNA binding activity of the intact ICP4 protein (26). In accordance, we found that an isolated ICP4 DNA binding domain including the i12 insertion (peptide I10i12X), produced no apparent band on either of the DNA probe fragments used in Figure 5. However, the I10i12X/VT4X heterodimers formed complexes with both the HSV-1 IE3 and the VZV gene 62 promoter probes, with intensities comparable to those formed by the VT4X homodimers. This result is consistent with the idea that in certain circumstances only one fully wild type monomer of the dimer is required for the interaction with DNA in a specific manner. Additionally, a previous study found that insertions at ICP4 residues 320 and 338 prevented DNA binding of the homodimer, but did not affect dimerization of the intact ICP4 protein (47). These data suggest that the C-terminal portion of region 1 and the N-terminal portion of region 2 are important for the DNA binding function rather than for dimerization.

I10X peptides containing insertion mutations at the majority of positions throughout ICP4 region 2 (i14–i18, see Fig. 6A), failed to bind to DNA as either I10X homodimers or as I10X/VT4X heterodimers (data not shown). However the simple interpretation that these insertions disrupt dimerization is arguable, as they may disrupt monomer interactions indirectly or inhibit DNA binding of the heterodimers, via gross conformational effects.

Insertions throughout the HSV-1 ICP4 DNA binding domain that disrupt DNA binding do not prevent dimerization

A major limitation of assaying heterodimer formation by gel retardation analysis is that only DNA binding competent heterodimers are detected. Mutational analyses indicate that ICP4 DNA binding activity is highly sensitive to minor alterations of the primary sequence (26,27). Therefore it is possible that some heterodimers which include a truncated or mutated monomer, fail to bind to DNA. Therefore the approach of co-immunoprecipitation of heterodimers was adopted to determine more stringently the effect of each insertion mutation on dimerization. Sequences spanning a heterologous epitope were fused to the ICP4 DNA binding domain, as described in the Materials and Methods section. The epitope, from the N-terminus of the HSV-1 ICP0 protein, is recognised very efficiently by monoclonal antibody 11,060 (44,45). The epitope-tagged ICP4 DNA binding domain is referred to as NXI10X (Fig. 6A).

Having established that the wild type I10X ICP4 DNA binding domain could heterodimerize with the NXI10X domain following *in vitro* co-translation, as determined by co-immunoprecipitation assay (not shown), the analysis was repeated with each insertion mutation version of the I10X domain. The insertions had no apparent effect on peptide solubility, as all remained in the soluble fraction after pre-clearing of the *in vitro* translated extracts (Fig. 6B). None of the insertions prevented dimerization with the epitope-tagged domain, as all were co-immunoprecipitated by MAb 11,060 (tracks 11–18, Fig. 6C). Additionally, the interaction was a consequence of specific heterodimerization as none of the insertion mutation peptides were co-immunoprecipitated when mixed with NXI10X after translation (tracks 3–10, Fig. 6C). It is apparent that DNA binding is more mutation sensitive than dimerization, as many of the insertions disrupted DNA binding (26, and see above).

Deletion of either the N- or the C-terminus of VZV 140k region 2 disrupts dimerization

Having found that the HSV-1 ICP4 and VZV 140k DNA binding domain peptides could readily heterodimerize, the co-immunoprecipitation approach was adopted using a panel of VZV 140k DNA binding domain deletion mutants. These peptides have sequential deletions into either the N- or C-terminus (Fig. 7A), deletions significantly encroaching on either end of region 2 (VT8, VT11, VT12) failed to form DNA binding competent heterodimers with an intact VT4X monomer (data not shown). The epitope-tagged HSV-1 ICP4 DNA binding domain peptide, NXI10X, was *in vitro* co-translated with each 140k deletion peptide and co-immunoprecipitations were performed on the pre-cleared *in vitro* translation extracts (Fig. 7B and C). The 140k DNA binding domain peptides VT2X, VT4X and VT5 specifically and strongly heterodimerized with the epitope-tagged domain (Fig. 7C, tracks 9–11). The VT2X peptide was also specifically co-immunoprecipitated in the presence of 1M NaCl (not shown), indicating that the interaction is not mediated by DNA and is highly stable. Peptides VT11 and VT12 (Fig. 7A), which have deletions into the N-terminal end of the 140k DNA binding domain, also heterodimerized (Fig. 7C, tracks 13 and 14), although at a lower efficiency than the VT2X peptide. Therefore, residues 504–647 (VT11) appear to be sufficient for dimerization, while truncation of the domain to residues 526–647 (peptide VT12) reduced dimerization activity yet further. Quantitation of the data found that VT11 and VT12

heterodimerized with the epitope-tagged domain at efficiencies of 15.3% and 5.7% (respectively) of that of VT2X. Co-immunoprecipitation of peptide VT8, which has a deletion into the C-terminal end of the VZV 140k DNA binding domain (Fig. 7A), was undetected even after prolonged exposures. We conclude that the N-terminal portion of VZV 140k region 2 (between residues 472 and 526) contributes to normal dimerization of the DNA binding domain whereas sequences in the C-terminal portion (between residues 588 and 647) are essential.

DISCUSSION

The results presented in this paper show that the highly purified DNA binding domain of the VZV 140k transcriptional regulator protein, is a stable dimer in solution and that it binds to DNA as a dimer. Moreover, it readily heterodimerizes with the corresponding domain of the HSV-1 IE protein, ICP4, suggesting that the structures and sequences mediating dimerization are conserved between these two homologous viral proteins. Comparison of the sequences bound by the homo- and heterodimeric proteins suggests that only one fully wild type subunit of the dimer is sufficient for specific DNA recognition in certain circumstances. Although the sequences required for the DNA binding interaction of the alphaherpesvirus protein DNA binding domains overlap with those required for the subunit interaction, deletion and insertion mutations are less disruptive to the later function.

The alphaherpesvirus regulatory proteins are closely related and the primary structures of HSV-1 ICP4, HSV-2 ICP4, VZV 140k, PRV IE180, EHV-1 IE1 and MDV ICP4 have been divided by extent of relative homology into five co-linear regions (24, B.Barnett pers. comm., 1,48–50). Sequences spanning the highly conserved region 2 of the HSV-1, VZV, and PRV protein homologues have been found to encode a DNA binding domain (29–32). These protein segments do not contain sequences that obviously conform to any of the recognised classes of DNA binding domain (51) and may well represent a novel class of DNA binding structure.

Like its HSV-1 counterpart, the DNA binding domain of VZV 140k DNA has an intrinsic dimerization activity and binds to DNA as a homodimer. We suggest that dimers of the 140k DNA binding domain are highly stable and have a low rate of subunit dissociation because: (a) no VT2 monomers were detected upon gel filtration analysis of the purified VT2 peptide (Fig. 3), and (b) heterodimer formation was not observed when the separately translated DNA binding domain peptides were mixed together (Figs. 4,5,6C,7C). The apparent high affinity of the monomer units for each other suggests that dimerization of the 140k DNA binding domain is likely to occur very rapidly after translation, perhaps even concurrently with the polypeptide folding process, as previously proposed for intact ICP4 (52).

Heterodimer formation between the VZV 140k and HSV-1 ICP4 DNA binding domain peptides occurred readily *in vitro* (Fig. 4B). It is likely that equivalent protein–protein interactions are involved in the formation of homodimers of the intact proteins during their respective infections. The protein interface mediating dimerization must be structurally similar in 140k and ICP4 to allow heterodimerization, and it is possible that the actual sequences involved in the interaction between monomers are conserved or semi-conserved between these two regulatory proteins.

The sizes of the mapped DNA binding domains of 140k and ICP4 are very large compared to the established classes of DNA binding domains, and it is likely that they contain distinct sequences mediating their dimerization and DNA recognition activities. In several of the defined classes of DNA binding domain, the amino acid sequences involved in dimerization are adjacent to the DNA recognition motif. Both the helix-loop-helix class of proteins (for example MyoD) (53), and the leucine zipper family of DNA binding proteins (for example GCN4) (54,55), have a basic DNA recognition motif followed by an α -helical region that provides a dimerization interface. Sequences strictly conforming to these or other established types of multimerization motifs are not found within the DNA binding domains of the VZV 140k family of alphaherpesvirus proteins. Mapping the sequences mediating DNA binding and dimerization requires the identification of mutations that disrupt DNA binding but do not affect dimerization; the insertion mutations in all but the most peripheral sequences of HSV-1 ICP4 region 2 that we analyzed were of this type. As no insertion mutations were identified that disrupted dimerisation, it is possible that the ICP4 DNA binding domain may be able to accommodate or compensate for the effects of the insertions upon the individual facets of the dimerization interface. Alternatively a short discrete sequence that has not been affected by the insertions may mediate dimerization. It is improbable that insertions i12 and i14 to i18 inclusive (into ICP4 sequences) are all directly disrupting DNA binding, and it is more likely that the specific structure of the domain required for DNA recognition is highly sensitive to minor conformational alterations.

The fact that dimers containing the VZV 140k VT11 and VT12 peptides were detected, albeit at a low level, suggests that deletion of the N-terminus of region 2 has indirect effects on the subunit interaction. In agreement, sequences between 140k residues 472 and 525 were not sufficient for dimerization, as seen from gel filtration analysis of a peptide comprising only these sequences (data not shown). Sequences in the C-terminus of 140k region 2 (lacking from peptide VT8) are essential for dimerization. A long tract of residues within these region 2 C-terminal sequences is highly conserved (approximately 80% identity) between VZV 140k and HSV-1 ICP4, and secondary structure prediction programmes suggest a highly α -helical nature of this tract. The well studied helix-loop-helix (53) and leucine zipper (54) dimerization motifs are almost totally α -helical, making the C-terminal portion of region 2 of these alphaherpesvirus proteins a particularly favourable candidate for mediating dimerization. However, a single amino acid mutational analysis of these conserved sequences in the ICP4 domain failed to identify any substitutions that disrupted dimerization (K.E.Allen and R.D.Everett, manuscript in preparation). It is possible that, as with the 4 amino acid insertions, the dimerization activity of the domain may tolerate minor changes of this type.

Heterodimers comprising VZV 140k peptide VT4X and a severely DNA binding compromised mutant ICP4 peptide (I10i12X) bind to DNA efficiently (Fig. 5). Additionally 140k/ICP4 DNA binding domain heterodimers bind to a probe for which the ICP4 peptide homodimer has an extremely low affinity (bottom panel of Fig. 5). These data suggest that only one fully wild type subunit of the dimer is sufficient for the specific interaction with the recognition site. This raises the possibility that both subunits of the dimer are able to bind DNA independently, however there is no evidence for this occurring; gel retardation experiments using probes of different sizes in

separate and mixed incubations gave no evidence of complexes containing both probe molecules (data not shown). The observation that in certain circumstances only one fully wild type subunit is sufficient for DNA binding could be explained by several (non mutually exclusive) models:

Only one subunit normally contacts the DNA binding site (Fig. 8A). The second monomer does not directly contribute to the specificity or affinity of the DNA binding interaction but stabilises the overall protein–DNA complex. Evidence suggests that both half sites of the ICP4 consensus are specifically recognised by the wild type homodimers (30,32,33,56). The lack of a direct repeat or any obvious dyad symmetry in the bipartite ICP4 consensus presents some difficulties for a model where monomers interact in an equivalent manner with each half of the binding site. In B form DNA the two half sites of the ATCGTCnnnn-YCGRC consensus (underlined) (34) are predicted to lie on the same face of the helix and could in theory be specifically contacted by a single monomer. In a similar scenario, the trimeric *Drosophila* heat shock factor (HSF) can form stable protein–DNA complexes in which only two of the three identical monomers contact the DNA (57).

Binding of the first monomer facilitates binding of the second monomer (Fig. 8B). The high affinity binding of the wild type monomer allows cooperative binding of the second monomer by reducing its energy requirement for DNA binding. In this case, the low affinity of the mutant monomer would not significantly reduce the DNA binding affinity of the heterodimer while the mutant homodimer would fail to bind DNA. The recognition site for the yeast GCN4 transactivator protein has non-equivalent half sites: the GCN4 interaction with the left half site is strong compared to the relatively weak right half site (58). The nature of this binding site facilitates cooperative binding of the second monomer of the GCN4 dimer to the weak half site, a similar situation may hold for the ICP4 and 140k binding sites.

Dimerization with a wild type monomer induces a conformational change in the mutant monomer (Fig. 8C). Binding to the wild type monomer induces the mutant monomer to adopt a DNA binding competent conformation either before or after binding to DNA. In the presence of DNA, many DNA binding proteins adopt stabilized structures, for example the λ Cro protein undergoes a large quaternary change when the dimer binds to its operator sequence (59). Structural changes in the wild type ICP4 monomer upon DNA binding may induce analogous conformational changes in the mutant ICP4 subunit, restoring its ability to specifically recognise target sequences.

However, heterodimers including a version of the ICP4 DNA binding domain containing insertion mutation i14, i15, i16, i17 or i18, failed to bind to DNA. If the outcome of these particular insertion mutations is to induce a profound conformational change on the domain, then it can be envisaged how the altered domain structure could prevent or destabilize heterodimer–DNA interactions occurring by any of the three models presented above.

The data presented here extends and confirms previous observations on the relatedness of the HSV-1 ICP4 and VZV 140k proteins. The ability to obtain large amounts of functional highly purified VZV 140k DNA binding domain from engineered *E.coli*, as described here, should facilitate future structural analyses and hopefully elucidate details of its dimerization and DNA binding interactions.

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